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PRODUCTION OF MIGRATION INHIBITION FACTOR (MIF) AND AN INDUCER OF PLASMINOGEN ACTIVATOR (IPA) BY SUBSETS OF T CELLS IN MLC[1]

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Supernatants of murine spleen cells activated in MLC contain both MIF and a factor which induces plasminogen activator secretion by macrophages. These activities are not found in supernatants of unstimulated T cells in control (syngeneic) cultures. Because no plasminogen activator or fibrinolytic activity was detected in the lymphocyte supernatants directly, but appeared when supernatants were added to macrophages, we infer that production of this activity is induced in macrophages by an inducer of plasminogen activator (IPA).

IPA and MIF activities are first detected after 24 hr of MLC; peak activity occurred on day 3. Both factors eluted from Bio-Gel P-100 columns in the range of Kd 0.15 to 0.25 (m.w. range 25,000 to 60,000 daltons).

Production of both factors in this system required the presence of T cells. In experiments using anti-Ly 1 and anti-Ly 2,3 sera + C, it was observed that both the Ly 1 (helper-type) and Ly 2,3 (cytotoxic/suppressor-type) T cell subpopulations produced each mediator. It is likely that the production of mediators by individual T cell subsets *in vivo* is not restricted by their intrinsic capabilities, but rather by factors which determine their activation. These results point to the possible importance of both T cell subpopulations in activating macrophages, and indirectly in aspects of tissue injury resulting from cell-mediated immune reactions involving protease activation.

Since IPA correlated with MIF in terms of activity (0.78 coefficient of correlation), kinetics of production, gel filtration characteristics, and production by T cell subsets, and because the assay for plasminogen activator is a sensitive and reproducible one, it should be increasingly useful in *in vitro* studies of cell-mediated immunity.

Cell-mediated immune reactions involve an interaction between sensitized lymphocytes, which determine the specificity of a reaction, and macrophages, which can present antigen and also be acted upon by the activated lymphocytes. A consequence of this interaction is the "activation" of macrophages, a term used to describe a variety of morphologic, biochemical, and functional changes (1, 2). Two properties associated with macrophages activated in this way are an inhibition in their migration and the secretion of neutral proteases, including plasminogen activator (PA)[4] (3, 4). Products of activated lymphocytes implicated in the process of macrophage activation include migration inhibition factor (MIF) (5, 6), macrophage activation factor (7), and an inducer of plasminogen activator (IPA) (8). It has been shown previously that while both B and T lymphocytes are capable of producing MIF (9, 10), its production by B cells appears, in general, to be T cell dependent (11, 12).

The present study was undertaken to explore the correlation between the production of MIF and the IPA in MLC. In addition, this system offered an ideal opportunity to analyze the capability of defined T cell subpopulations, i.e., Ly 1 helper-type T cells and Ly 2,3 cytotoxic/suppressor-type T cells to produce these mediators, since both subpopulations can be activated in MLC.

MATERIALS AND METHODS

Animals. NCS mice (Rockefeller University) were used as a source of unstimulated peritoneal macrophages for the PA assay. C57BL/10J (B10), B10.D2/nSn (B10.D2), DBA/2J (DBA/2), and B6D2F$_1$/J (BDF$_1$) mice (Jackson Laboratories, Bar Harbor, Maine) were used to prepare spleen cell supernatants. BALB/cJ (BALB/c) mice (Jackson Labs) were used as a source of macrophages for the MIF assay.

Mastocytoma P815 cells. These were maintained by serial passage in DBA/2 mice. These were used for immunization of B10 mice and as ^{51}Cr-labeled targets in the cytotoxicity assay. Ten million cells were injected i.p. into B10 mice and spleen cells were collected 4 to 16 weeks later as a source of primed spleen cells.

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[3] S. G. is a Scholar of the Leukemia Society of America.

[4] Abbreviations used in this paper: MIF, migration inhibition factor; NMS, normal mouse serum; subscript m, mitomycin C treatment; D-MEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; ATDS, acid-treated dog serum; DTH, delayed-type hypersensitivity; IPA, inducer of plasminogen activator; 2-ME, 2-mercaptoethanol; PEC, peritoneal exudate cell; PA, plasminogen activator.

Mixed lymphocyte cultures. Primary two-way MLC were set up with B10 and either DBA/2 or B10.D2 spleen cells. One-way secondary MLC consisted of primed B10 spleen cells restimulated *in vitro* with either DBA/2_m[4] or BDF_1 spleen cells. In secondary MLC, responder cells (B10) were first passed over nylon wool columns (13) and the nonadherent (T-enriched) fraction used as responders. In some experiments untreated DBA/2 spleen cells were used in secondary MLC; hence in such cultures a concomitant primary MLC is also present. MLC were set up in 16-mm Linbro wells containing 5×10^6 nylon wool purified responder cells and 5×10^6 stimulator cells in 2 ml. In one-way MLC, the latter were treated first with mitomycin C (Sigma Chemical Co., St. Louis, Mo.) 50 μg/ml at 37°C for 30 min, washed twice with Hanks' balanced salt solution, (HBSS) and resuspended in medium. Culture medium was RPMI 1640 containing penicillin (200 I.U./ml), streptomycin (100 μg/ml), L-glutamine (0.3 mg/ml), 5×10^{-5} M 2-mercaptoethanol (2-ME) and 2% heat-inactivated (56°C, 30 min) FCS. Cultures were incubated in a humidified atmosphere of 7% CO_2, 93% air. Supernatants of MLC were collected at intervals, Millipore filtered (0.45 μm) to remove cell fragments that might interfere with the assay for PA, and used directly for the PA assay or adjusted to 12% serum and assayed for MIF activity. Controls consisted of supernatants from stimulator and responder cells cultured alone; in some experiments, these were combined.

PA assay. This assay was carried out as previously described (14). Briefly, I^{125}-fibrinogen-coated Linbro wells were preactivated in Dulbecco's modified Eagle's medium (D-MEM), 10% FCS at 37°C, and washed twice with HBSS. Unstimulated adherent peritoneal cells (5×10^5) were incubated in each well with 0.4 ml of dilutions of test supernatants for periods of up to 3 days. Culture medium alone was always monitored for solubilization of fibrin. Thereafter, cells were washed twice with HBSS and incubated with 0.5 ml of D-MEM acid-treated dog serum (ATDS) as a source of plasminogen. At intervals from 90 to 360 min, 0.1 ml of supernatant was collected to assess radioactivity released by macrophages. Cultures were examined by inverted phase contrast microscope at all stages of the assay.

MIF assay. The assay for MIF was carried out with peritoneal exudate cells (PEC) harvested from BALB/c mice given 3 ml of Marcol '52 i.p. 4 days previously. Cells were harvested, washed twice with HBSS, and resuspended in RPMI 1640 containing 12% FCS and antibiotics. Cells were packed into capillary tubes and placed two per chamber. Each supernatant was tested in two chambers. After 24 hr, areas were projected and migration inhibition calculated as percentage of migration in control supernatants.

^{3}HTdR incorporation. Thymidine incorporation in MLC was assessed at day 3 in flat bottom microtiter plates by addition of 1 μCi ^{3}HTdR (sp. act. 3.0 Ci/mmole) 16 to 24 hr before harvest. Microtiter wells, in triplicate, contained 2×10^5 stimulator and an equal number of responder cells in 0.2 ml. Cultures were harvested with a Skatron Multiple Cell Culture Harvester onto glass fiber filters, dried, placed in scintillant (NEN No. 950A), and counted in a Beckman Liquid Scintillation Counter.

Cytotoxicity assay. Cytotoxic effector cells generated in II° MLC were measured on day 3 in a 16-hr assay at a 40:1 ratio effector:targets with ^{51}Cr-labeled P815 cells as targets, as previously described (15). Spontaneous release value was 25%. Total releasable counts were determined by three cycles of freezing and thawing. Essentially similar results were obtained in a 4-hr assay. Results are expressed according to the formula:

$$\% \text{ specific lysis} = \frac{\text{exptl.cpm released-cpm released spont.}}{\text{total releasable cpm} - \text{cpm released spont.}} \times 100$$

Antisera. Anti-Thy 1.2 serum (AKR anti-C3H) was used at a final dilution of 1:10; BDF_1 serum was used as a source of normal mouse serum (NMS). Whole spleen cells from P815-primed B10 mice were treated with anti-Thy 1.2 serum, NMS, or left untreated. Cell lysis was monitored by trypan blue exclusion. After serum treatment, cell suspensions were washed twice and reconstituted to original volume and placed in MLC with B10.$D2_m$ stimulator cells. In experiments with Ly antisera, 20×10^6 nylon wool purified P815-primed B10 spleen cells were incubated with a 1:6 dilution of either anti-Ly 1.2 or anti-Ly 2.2 serum prepared as described previously (16, 17) or NMS. Ly sera were absorbed as described (16) with congenic thymocytes to render them monospecific, and their specificity was confirmed in functional assays before use. After 30 min at 4°C, cells were washed once with HBSS and resuspended in medium containing rabbit C at 1:8. Cells were incubated at 37°C for 40 min with rocking. Results were assessed by exclusion of trypan blue. Cells were washed twice and resuspended either to 5×10^6/ml or in some experiments to original volume, to assess the function of the surviving cells without enrichment relative to their proportions in the T cell populations before lysis with antisera, and MLC were initiated as described.

Gel filtration. Column fractionation of control and MLC supernatants was carried out as follows. Supernatants (120 ml) in 2% FCS were dialyzed twice against saline and twice against distilled water, freeze dried, reconstituted in PBS, and applied to Bio-Gel P-100 columns (4°C) calibrated before and after each run with a mixture of human γ-globulin, crystalline bovine serum albumin, ovalbumin, chymotrypsin, and cytochrome C. Nine-tenths milliliter/tube was collected, fractions I through V were pooled, dialyzed against distilled water, freeze dried, reconstituted with medium, Millipore filtered, and assayed for MIF and IPA activities.

RESULTS

Production of an IPA by MLC supernatants. Unstimulated macrophages were exposed to MLC supernatants (25% v/v) for 3 days, washed, and assayed for fibrinolytic activity. As seen in Figure 1, there was a 5-fold stimulation of fibrinolysis by macrophages which had been incubated with MLC supernatants, whereas control supernatants and medium alone had no observable effect. These supernatants contained no detectable fibrinolytic activity. In addition, no fibrinolysis occurred in the absence of plasminogen; the addition of purified dog plasminogen restored the activity, indicating that the supernatants contained an IPA.

Table I illustrates the reproducibility of production and measurement of IPA in MLC supernatants. In row A, a supernatant

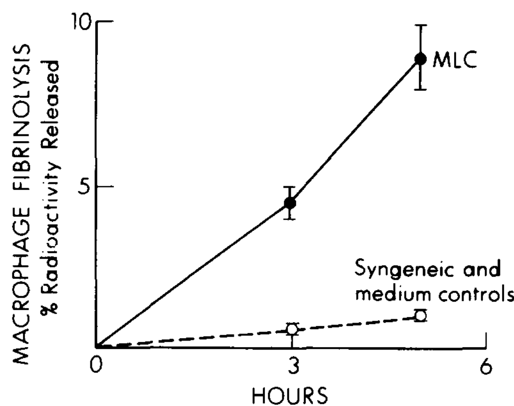

Figure 1. Induction of macrophage fibrinolysis by MLC and control supernatants. In these and subsequent experiments, the total releasable counts was always equal to or greater than 50,000 cpm.

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TABLE I

Enhancement of macrophage fibrinolysis by MLC supernatants

	No. of Expts.	Fibrinolytic activity [a]			
		Medium alone	DBA/2	B10	MLC
A. Repeated assays	7	1.3 ± 0.31	2.9 ± 0.74	2.0 ± 0.64	9.5 ± 0.40
B. Primary MLC	5	0.9 ± 0.15	1.4 ± 0.40	1.3 ± 0.17	5.7 ± 1.10
C. Secondary MLC	7	1.2 ± 0.25	1.0 ± 0.15	1.2 ± 0.08	7.3 ± 0.67

[a] Percentage of radioactivity released in 3 hr/0.1 ml supernatant (final concentration 25% v/v) means ± S.E.

of a I° MLC, stored at −70°C in aliquots, was tested seven times over a 3-month period and showed no detectable loss in activity. In rows B and C are shown results from separate experiments with supernatants of either I° MLC (B) or II° MLC (C). Again, results were highly reproducible; MLC fractions stimulated a 6- to 7-fold increase in IPA activity over medium alone, whereas control supernatants showed only a 1- to 2-fold increase. II° MLC showed a slight increase in IPA activity compared to I° MLC.

Macrophages exposed to MLC supernatants after 1 day demonstrated increased spreading, membrane ruffling, and prominent cytoplasmic granules. Enhanced fibrinolysis was not due to differential adherence of macrophages incubated with MLC supernatants. On occasion, active fractions were toxic and consequently fibrinolytic activity was diminished. This toxicity was avoided by testing dilutions of the supernatant. Active supernatants induced secretion of PA within 24 hr. This increased somewhat with time and was maximal on day 3, which was then chosen as the standard induction period for most studies.

Dose response curves indicated that IPA activity was linear in the concentration range of 10 to 25% v/v with no further increase at higher concentrations. It was important to use 2% FBS during the induction phase, because of the presence of inhibitors. IPA activity was also generated in the presence of 5 to 10% heat-inactivated horse serum.

Relation between IPA and MIF production. Since MIF activity is also generated in MLC, we investigated the relationship of migration inhibition to production of IPA. Figure 2 illustrates that MLC generated MIF as well as IPA activity. The two activities were closely correlated in time; both were first detected on day 2 and were maximal on day 3. IPA activity was not detected in syngeneic controls until day 4. This close correspondence was noted in 26 separate experiments, with a correlation coefficient of 0.78 (95% confidence limits ± 0.11).

Mediator production by T cells and T cell subsets. It was important to establish the nature of the cell type responsible for production of both of these mediators in this system. Both activities were abolished by treatment of the responding cells with anti-Thy 1.2 serum plus C, but not by NMS plus C, as shown in Table II. As a control it was demonstrated that T cell-mediated cytotoxicity (for P815 cells) was similarly abolished by treatment with anti-Thy 1.2 serum plus C.

In vivo primed responder cells were pretreated with anti-Ly sera + C before II° *in vitro* MLC to define the ability of the various T cell subpopulations to produce these mediators. Figure 3 shows the results of one experiment (repeated twice) in which the Ly 1 subpopulation, which retained no significant cytotoxic activity, produced approximately 50% of the IPA and MIF activities of the untreated responding cells. Similarly, cultures of spleen T cells depleted of Ly 1 cells showed no significant uptake of ^{3}HTdR, yet retained 70 to 80% of IPA and MIF activities. Reconstitution of the two subpopulations restored full MIF and cytotoxic activities, 60% of the IPA activity, and 50% of the ^{3}HTdR incorporation. We have confirmed these observations by using BDF_1 stimulator cells in place of DBA/ 2_m cells to eliminate the possibility that the stimulator cells may produce these mediators.

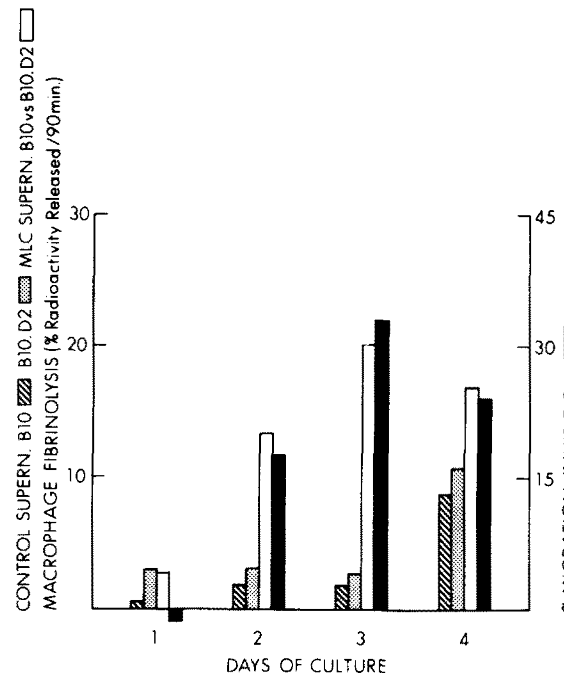

Figure 2. Kinetics of appearance of IPA and MIF activities in spleen cell cultures. Cumulative supernatants were collected from primary two-way MLC (B10 *vs* B10.D2) and controls.

TABLE II

Production of MIF and IPA in MLC requires the presence of thymus-derived lymphocytes

	Fibrinolysis % Radioactivity Released/5 hr	% Migration Inhibition	Cytotoxicity % Specific Lysis
MLC, untreated	7.7	40	79
Anti-Thy 1.2 + C	1.7	0	1
NMS + C	6.3	42	57
Medium control	1.4	0	

Gel filtration of spleen cell supernatants. Because of the similarities in the kinetics of appearance and cell source, we attempted to separate these factors on columns of Bio-Gel P-100. Figure 4 shows that both activities eluted together and were maximal in fractions III and IV with K_d values of 0.15 to 0.25, corresponding to a m.w. range of 25,000 to 60,000 daltons.

DISCUSSION

The MLC system was selected for assessment of mediator production and analysis of T cell subsets involved in their production for three reasons. The system obviates the necessity of reconstituting antigen or mitogen to control supernatants, which, upon concentration, are a potential source of difficulty. More importantly, MLC generated with spleen cells of mice differing at the I and K + D regions of the H2 complex are known to generate high levels of proliferating and cytotoxic T

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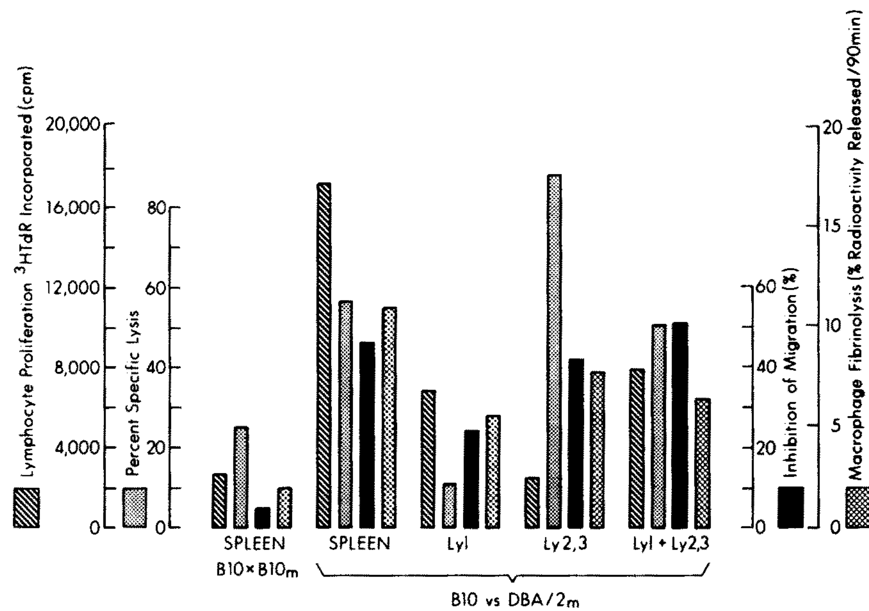

Figure 3. Production of MIF and IPA by subsets of T cells in secondary one-way MLC.

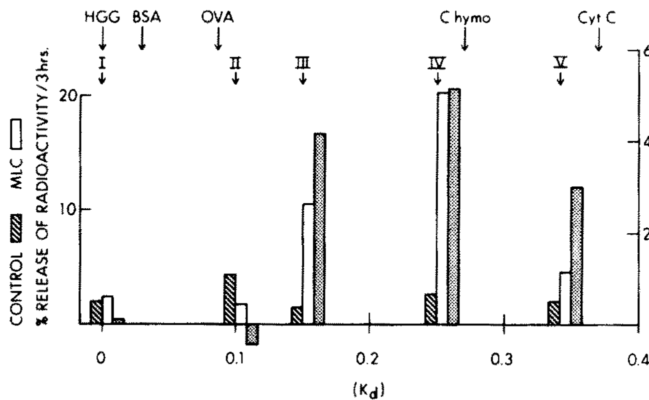

Figure 4. Fractionation of MLC and control supernatants on Bio-Gel P-100. Roman numerals point to the partition coefficient (K_d) for each of the five fractions assayed from MLC and control supernatants.

lymphocytes, whereas Con A may activate only the former type (18). The use of anti-Ly sera therefore permits assessment of the contribution of each subpopulation to the process of macrophage activation mediated by soluble T lymphocyte products. Lastly, this system may serve as a useful model of the graft-*vs*-host reaction, in which it is likely that activated macrophages participate in mechanisms of immunologically induced tissue injury.

We have demonstrated that primary and secondary MLC generate both MIF and an IPA. Since no fibrinolytic activity was detected in the stimulated lymphocyte supernatants, the production of PA is clearly a process induced in the macrophages. In addition, the enzyme(s) produced by these *in vitro* activated macrophages requires the presence of plasminogen as substrate to exhibit fibrinolysis, indicating enzymic specificity. These results are consistent with previous observations that PA activity can be induced in macrophages treated with supernatants of mitogen-activated (8) or antigen-activated (19, 20) lymphocytes in the mouse and guinea pig. In the present study, as has been demonstrated in another system (19), production of IPA is dependent upon the presence of thymus-derived lymphocytes.

The production of the IPA and MIF was closely correlated in this system. Both activities were detected at 24 hr after lymphocyte stimulation, and were maximal on the 3rd day. Both factors eluted in the m.w. region of 25,000 to 60,000 daltons on Bio-Gel P-100 columns, in general agreement with previous reports on mouse MIF (21). More extensive chemical fractionation will be required to resolve whether MIF and IPA activities are truly mediated by the same moiety. In any case, the sensitivity of the PA assay and the high degree of correlation with MIF activity suggest that this assay should be increasingly useful in studies of macrophage activation and cell-mediated immunity.

Whereas helper and cytotoxic activities appear to be specialized functions of distinct subpopulations of T cells, it appears that production of MIF, IPA, and possibly other mediators is likely to be carried out by both cell populations, at least in the MLC system. Splenic T cells from mice primed against alloantigens, depleted of either Ly 1^+ or Ly 2^+ cells, were capable on restimulation *in vitro* of generating MIF and IPA activities. These results are consistent with those of Miller *et al.* (22) who demonstrated that lymphocytes from DNCB-sensitized mice were capable of transferring the cell-mediated contact hypersensitivity reaction if the recipients shared either I, K, or D genes with the donor, suggesting that both Ly 1 and Ly 2,3 cells were involved. In the case of protein antigens, I-region compatability alone was required. A reasonable interpretation of these data is that in the case of protein antigens presentation occurs in association with I-region gene products on the macrophage, and is likely to engage primarily if not exclusively the Ly 1 type T cell. In the case of DNCB, which is capable of conjugating to cell membrane antigens, and in the case of the related hapten TNBS, which has been shown to modify gene products of the H-2 complex (23, 24), presentation would occur both by macrophages and by direct contact with modified H2 engaging both Ly 1 and Ly 2,3 cell populations. This is consistent with the results of Nagy *et al.* (25) who showed that binding of I region gene products or K region gene products by T cells blasts activated to both I and K region differences was a property of the Ly 1 and Ly 2,3 subpopulations, respectively. We would interpret the findings of Huber *et al.* (26) that only Ly 1^+ and not Ly $2,3^+$ cells could transfer delayed-type hypersensitivity (DTH) to SRBC to indicate that, for whatever reason, that antigen fails to engage the Ly $2,3^+$ cytotoxic population.

It thus appears likely that mediator production is a general property of activated T (as well as B) lymphocytes, and that

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the type of T cell subpopulation engaged in mediator production is likely to be controlled more by the nature of antigen presentation than by the intrinsic capability of the cell population. An interesting test of this hypothesis would be to assay supernatants of Ly 1 and Ly 2,3 cells activated in MLC for soluble immune response suppressor activity, since Tadakuma *et al.* (27) have shown that this factor, which suppresses an *in vitro* PFC response to SRBC, is inseparable from MIF by several physical criteria.

Although it is clear that active supernatants induce rather than possess PA activity, it is still problematic whether MIF acts directly on macrophages to inhibit their migration, or whether it induces additional factors that interact with medium components, for example, serum proteins, to produce the molecule(s) that ultimately regulates the migration of the macrophage. The recent report of Roblin *et al.* (28) is particularly relevant since a MIF-like activity could be produced by the action of PA on serum. It is important to establish whether this mechanism of migration inhibition mediated by enzyme-treated serum proteins or serum proteins acted upon by products of transformed cells is identical to the mechanism(s) of antigen-induced inhibition of migration.

T cell induction of enzyme secretion by macrophages, PA in this case, may point to a role for immune amplification of a number of enzyme-dependent systems involved in inflammation, tissue catabolism, coagulation complement, and the kinin systems (29–33). Recent experiments in this laboratory have indicated that neutral proteases in macrophage supernatants, including PA, initiate striking degradation of basic protein in purified myelin (34). Hence, T cells may serve a catalytic function amplified by a variety of enzymes and cell types leading to localization of infection or tissue injury.

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